Asian Pac J Trop Med 2014; 7(Suppl 1): S212-S216

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi: 10.1016/S1995-7645(14)60234-0

Characterization of cephalosporin-resistant clinical Enterobacteriaceae for CTX-M ESBLs in Bahrain

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ARTICLE INFO

Article history:
Received 5 Jun 2014
Received in revised form 18 Jun 2014
Accepted 13 Jul 2014
Available online 12 Sep 2014

Keywords:
Extended-spectrum beta-lactamases
class A ESBLs
CTX-M-15
CTX-M-55
Bahrain

ABSTRACT

Objective: To detect the presence of specific CTX-M class of extended spectyum β-lactamases (ESBLs) in a collection of cephalosporin-resistant Enterobacteriaceae isolates from Bahrain.

Methods: A subset of 80 cephalosporin-resistant Enterobacteriaceae collected from Salmaniya Medical Complex, Bahrain, were characterized further for the presence of specific genogroups of CTX-M β-lactamases by multiplex- and monoplex- PCRs. The primers used for the multiplex and monoplex PCRs were of genogroups- 1, 2, 8, 9 and 25. Sequencing of the representative isolates was performed to find the circulating CTX-M-types.

Results: A total of 93.8% (75/80) isolates showed the amplicons corresponding to any of the genogroups (1, 2, 8, 9, 25) and the remaining 6.2% isolates turned out negative in multiplex PCR. Some of the isolates demonstrated multiple bands corresponding to the sizes of different genogroups. Further confirmation with respective monoplex PCR on these 75 isolates demonstrated that 93.3% (70/75) harbored CTX-M genogroup-1 and 6.7% (5/75) harbored genogroup-9. We did not find the presence of genogroups 2, 8, and 25 in these isolates by monoplex PCR. Sequencing results of genogroup-1 isolates demonstrated the presence of CTX-M-15-like ESBL, however, discrepant results were noticed in genogroup-9 isolates, sequencing showed them as CTX-M-55-like ESBL.

Conclusions: This is the first report from Bahrain characterizing the CTX-M genogroups of ESBLs and reporting the emergence of $bla_{\text{CTX-M-55}}$-like gene in this region.

1. Introduction

The variants of CTX-M extended-spectrum β-lactamases (ESBLs) have now been circulating for more than 2 decades since its first appearance in 1990[1]. Currently these CTX-M-types are approaching 150 in number (CTX-M-1 to CTX-M-151), however, few CTX-Ms such as CTX-M-14 and CTX-M-18, CTX-M-55 and CTX-M-57, CTX-M-2 and CTX-M-97, and CTX-M-3 and CTX-M-133 were reported as identical (http://www.lahey.org/Studies/other.asp#table1; last accessed)[2]. The increasing use of successive generations of β-lactam antibiotics has put a selective pressure on the bacterial population to produce numerous β-lactamases, including the CTX-M ESBLs and thus leading to resistance against cephalosporins[3]. This rising resistance to the third- and fourth-generation cephalosporins has forced to increase the use of carbapenems in clinical settings. However, due to the increasing usage of carbapenems in recent years, the

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Foundation Project: Supported by Research and Ethics Committee and the College of Medicine & Medical Sciences, Arabian Gulf University (Grant No. 94).

resistance to this group of antibiotics has also appeared[3,4].

In response to the global call towards the rising threat of antimicrobial resistance (AMR), emphasis is now being put on development of surveillance programs in individual countries. To that end, regional scientific studies are badly needed, especially the research studies addressing the issues of AMR at a molecular level.

While performing a careful search of literature on PubMed, we could get only one molecular study from Bahrain[5], which too didn't characterize the genogroups (CTX-M clusters) or the genotypes (CTX-M types) of this important class of ESBLs, the CTX-M ESBL. Therefore, we planned this extensive molecular study to characterize the Bahraini Enterobacteriaceae for the presence of specific genogroups and genotypes of CTX-M enzymes.

2. Materials and methods

2.1. Bacterial isolates

The bacterial cohort comprised of 80 Enterobacteriaceae isolates [75 *Escherichia coli* (*E. coli*) and 5 *Klebsiella pneumoniae* (*K. pneumoniae*)] that were previously tested for the presence of CTX-M genes by using the general set of primers; the isolates were not characterized for CTX-M genogroups or the CTX-M types in that study[5]. These isolates were collected from a 1 000-bed secondary care hospital, Salmaniya Medical Complex, from Bahrain during the period 1st January to 30th June 2006.

2.2. Molecular characterization

Molecular studies were carried out in the Department of Microbiology, Immunology & Infectious Diseases (DMIID) of the College of Medicine & Medical Sciences of Arabian Gulf University, Bahrain. The isolates were subjected to multiplex- and monoplex-PCR reactions according to the protocol described elsewhere[6]. The sets of primers used for the multiplex PCR were of the following genogroups: bla_{CTX-M} group-1, bla_{CTX-M} group-2, bla_{CTX-M} group-8, bla_{CTX-M} group-9 and bla_{CTX-M} group-25. The details of the primers used and the expected amplicons sizes of the respective CTX-M genogroups are shown in Table 1. Following cycling conditions were used for the detection of bla_{CTX-M} genogroups by multiplex-PCR: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 60 seconds, initial extension at 72 °C for 1 min and with a final extension at 72 °C for 10 min. For bla_{CTX-M} monoplex-PCR the cycling conditions were: initial denaturation at 94 °C for 7 min, followed by 35 cycles consisting of 94 °C for 50 seconds, 50 °C for 40 seconds and 72 °C for 2 min and a final elongation at 72 °C for 5 min.

The isolates found positive for any multiplex-PCR reaction were classified into the genogroups based on the amplicon sizes and were further subjected to a monoplex-PCR reaction for confirmation using the sets of primers of the respective genogroups. In the isolates where multiple bands corresponding to different genogroups were noticed, they were subjected to separate monoplex-PCR reactions using the respective primer sets.

Table 1

The list of primers used for multiplex- and monoplex-PCRs and the expected amplicons.

Target gene	Primer used	Primer sequence	Amplicon size
bla_{CTX-M} group-1	CTX-M gp1F	5′-AAA AAT CAC TGC GCC AGT TC-3′	415 bp
	CTX-M gp1R	5′-AGC TTA TTC ATC GCC ACG TT-3′	
bla_{CTX-M} group-2	CTX-M gp2F	5′-CGA CGC TAC CCC TGC TAT T-3′	552 bp
	CTX-M gp2R	5′-CCA GCG TCA GAT TTT TCA GG-3′	
bla_{CTX-M} group-9	CTX-M gp9F	5′-CAA AGA GAG TGC AAC GGA TG-3′	205 bp
	CTX-M gp9R	5′-ATT GGA AAG CGT TCA TCA CC-3′	
bla_{CTX-M} group-8	CTX-M gp8F	5′-TCG CGT TAA GCG GAT GAT GC-3′	666 bp
bla_{CTX-M} group-25	CTX-M gp25F	5′-GCA CGA TGA CAT TCG GG-3′	327 bp
	*CTX-M gp8/25R	5′-AAC CCA CGA TGT GGG TAG C-3′	

*: This primer was used as a reverse primer for genogroup-8 and genogroup-25.

2.3. Sequencing of the bla genes from representative isolates

Once the *bla* genes were characterized into CTX-genogroups based on multiplex- and monoplex-PCR experimentations, representative isolates from the different CTX-M genogroups were further subjected for sequencing experimentations in order to identify the CTX-M genotypes. The amplicons were subjected to purification experimentation at DMIID and further submitted to Genoscreen, France for sequencing. The primers used in the monoplex-PCR for respective genogroups were used for the sequencing reactions. The sequence results thus obtained from the Genoscreen were analyzed at DMIID by using the Chromas software (http://technelysium.com.au/)[7], and the basic local alignment search tool (BLAST) search engine. Further alignment was performed against the specific CTX-M-types by using the Clustal W software (http://www.genome.jp/tools/clustalw/)[8].

3. Results

In multiplex-PCR, a total of 93.8% (75/80) showed the amplicons corresponding to any of the CTX-M genogroups (1, 2, 8, 9, and 25) and the remaining 5 (6.2%) isolates turned out negative despite repeated attempts. Seventy *E. coli*, out

of 75 isolates, showed the amplicons in PCR while 5 isolates didn't show any. All the 5 *K. pneumoniae* isolates showed amplicons in the PCR reactions. Some of the isolates demonstrated multiple bands corresponding to the sizes of different genogroups. The detailed results are shown in Figure 1.

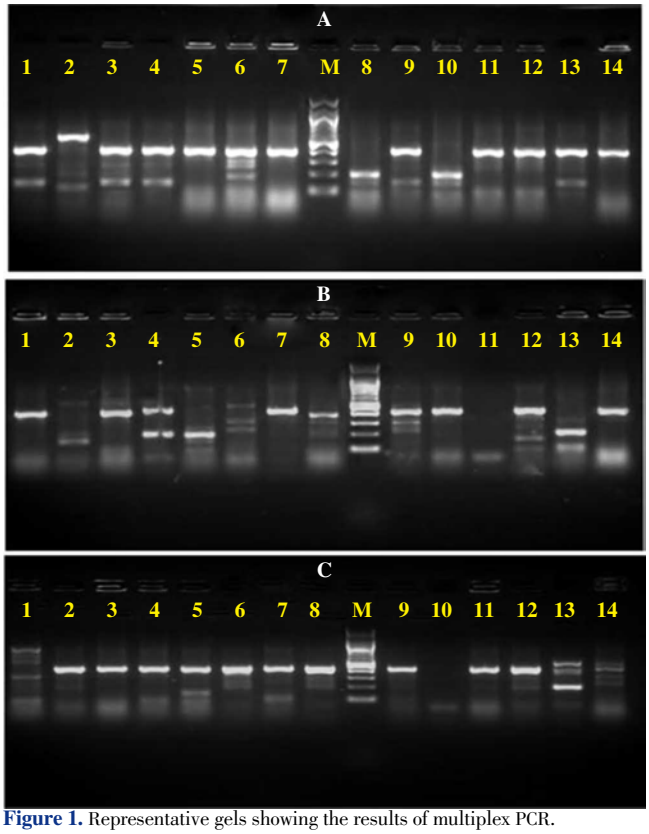

Figure 1. Representative gels showing the results of multiplex PCR.
A: Lanes 1, 3-7, 9, 11-14 show amplicons of 415 bp corresponding to CTX-M genogroup-1; Lane 2 shows amplicon of 552 bp corresponding to CTX-M genogroup-2; Lanes 8 and 10 show amplicons of 205 bp corresponding to CTX-M genogroup-9. B: Lanes 1, 3, 4, 7-10, 12, 14 demonstrate amplicons of 415 bp corresponding to CTX-M genogroup 1; Lanes 4, 5 and 13 show amplicons of 205 bp corresponding to CTX-M genogroup 9, kindly note that lane 4 shows 2 amplicons of multiplex PCR (415 bp and 205 bp) corresponding to CTX-M genogroup-1 and CTX-M genogroup-9. C: Lanes 2-9 and 11, 12 show amplicon of 415 bp for CTX-M genogroup-1; Lane 13 shows multiple bands and lanes 1 and 14 were negative (nonspecific amplification). Lane M in the gels demonstrates 100 bp ladder.

3.1. Determination of bla_{CTX-M} genogroups by PCR

The isolates were grouped into genogroups according to the respective amplicons sizes (kindly refer to Figure 1 and Table 1). Further confirmation with respective monoplex-PCR on these 75 isolates demonstrated that 93.3% (70/75) harboured CTX-M genogroup-1 (Figure 2A) and 6.7% (5/75) harboured genogroup-9 (Figure 2C). Of the 70 CTX-M genogroup-1 harbouring isolates, 65 were *E. coli* and 5 were *K. pneumoniae*, whereas all the genogroup-9 harbouring isolates (n=5) were *E. coli*. One of the isolates that showed the presence of genogroup-9 also demonstrated the presence of a band corresponding to CTX-M genogroup-1, however, on performing the monoplex-PCR with genogroup-1 primers, it turned out negative for genogroup-1 (Figure 1B). We did not find the presence of genogroups-2, 8, and 25 in these isolates by monoplex PCR (Figure 2B).

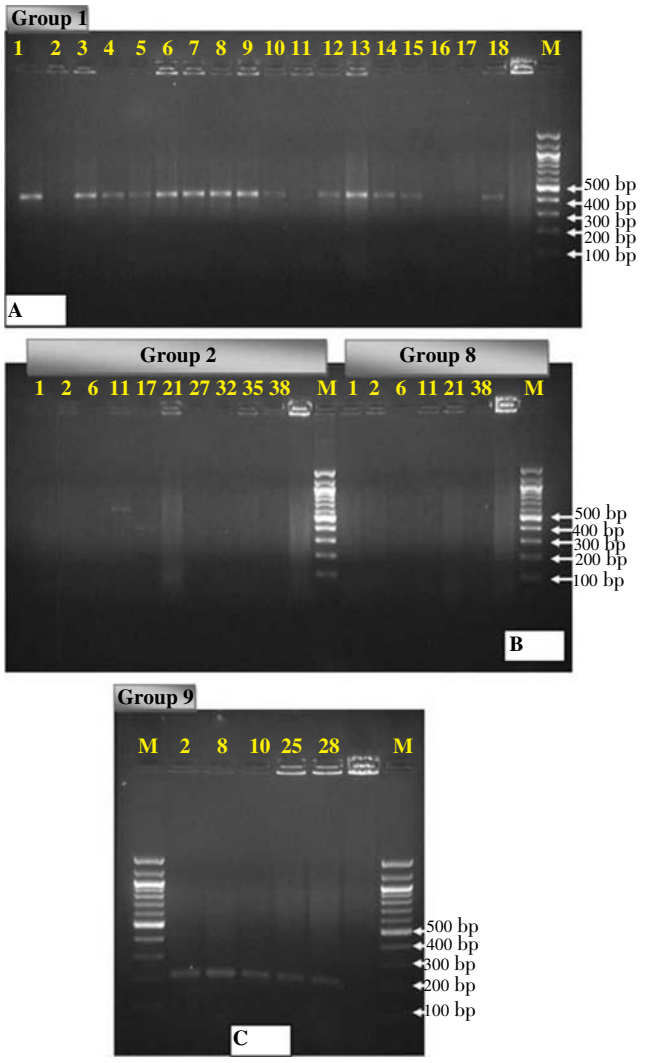

Figure 2. Representative gels showing the results of monoplex PCR.
A: Representative results of the monoplex PCR done for CTX-M genogroup-1; B: Representative results (negative) for CTX-M genogroup 2 and CTX-M genogroup 8 in monoplex PCRs; C: Representative results of monoplex PCR for CTX-M genogroup-9; Lane M in all the gels demonstrates 100 bp ladders.

3.2. Determination of CTX-M types by sequencing

Thirteen representative isolates (10 from CTX-M genogroup-1 and 3 from CTX-M genogroup-9) were subjected for sequence analyses. On performing the BLAST search on isolates belonging to CTX-M genogroup-1, 100% similarity was found with CTX-M-15 type ESBL. Alignment of the sequence with a known CTX-M-15 sequence (Accession No. KF769131.1) showed a perfect match. The BLAST search on CTX-M-9 genogroup harbouring isolates showed 99%

similarity with CTX-M 55-type ESBL and the alignment using a known CTX-M-55 sequence (Accession No. KC576516.1) showed a perfect match.

4. Discussion

ESBLs are the enzymes that confer resistance against broad-spectrum cephalosporins, including third- and fourth-generation cephalosporins, and aztreonam; this third- and fourth-generation cephalosporins resistance has become a major concern worldwide[3,9,10]. The wider spread of these ESBLs in clinical Enterobacteriaceae has posed a serious AMR threat during the last few decades[11]. The ESBL phenotypes have been reported from the Middle East region, including from Bahrain[12-15]. However, the studies characterizing the CTX-M genogroups and CTX-M-types at a molecular level (especially by sequencing) are fragmentary from this region. Moreover, there is a complete lack of such studies from Bahrain.

The CTX-M types of ESBLs are currently the widespread enzymes globally and its variants are continuously increasing in numbers, CTX-M-15 being the widely prevalent type[1,6,16]. CTX-M-15 has also been reported in few studies from Arab countries, the first description being from Egypt and followed by from United Arab Emirates, Kuwait and Saudi Arabia[12,17-20]. In the present study, majority of the isolates (93.3%; 70/75) were of CTX-M genogroup-1 and the sequencing of the representative isolates demonstrated the presence of CTX-M-15 type. This signifies that CTX-M-15 is the prevalent CTX-M type in this part of the world, including in Bahrain.

A minority of the *E. coli* isolates (n=5) demonstrated an amplicon corresponding to the CTX-M genogroup-9, however, sequencing of the representative isolates designated them as CTX-M-55-like ESBL, an enzyme belonging to the CTX-M-1 cluster. This discrepancy in the PCR and sequencing results is noteworthy and suggests that sequencing should always be performed to designate the specific CTX-M types. The CTX-M-55 was first reported in Enterobacteriaceae from Thailand[21], and thereafter this enzyme has been reported from various countries, most of the reports being from China[22-25]. The CTX-M-55 is reported to differ from CTX-M-15 by a single amino acid substitution at position 80 (Ala80Val) and could be an evolutionary process due to the antibiotics selective pressure[22]. CTX-M-15 enzymes exert increased catalytic activity against cefotaxime than ceftazidime, whereas the CTX-M-55 enzymes have activity against both of these antibiotics[22]. While looking to the antibiotics data retrospectively, we noticed that all these 5 isolates had resistance against both cefotaxime and ceftazidime. We didn't find any published report regarding the occurrence of this CTX-M type (CTX-M-55) from the Arab countries. The occurrence of the CTX-M-55 in this region, though in a smaller bacterial population, suggests the bacterial evolution in response to the selection pressure that could be a consequence of over prescription or weak enforcement of the existing antibiotics policies. We also wish to emphasize that the CTX-M-55 like *bla* genes have already emerged in Kingdom of Bahrain at least 8 years ago, as speculated by their presence in a bacterial collection from year 2006. Moreover, this pilot study also suggests a need to look for such genes in recent, as well as in collections prior to year 2006 (if available with any research group), so as to shed more light on their likely time of emergence and also the current situation.

In nutshell, CTX-M-15 is the prevalent ESBL in Bahraini Enterobacteriaceae; however, CTX-M-55 has emerged, though in a minor proportion. As evident from our study, multiplex/monoplex-PCRs alone should not be performed in order to report the emergence of new variant of ESBL, rather the results should be confirmed by sequencing. To the best of our knowledge, this is the first report from Bahrain characterizing the CTX-M genogroups of ESBLs and reporting the emergence of $bla_{\text{CTX-M-55}}$-like gene in this region.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

M. Shahid is thankful to Arabian Gulf University, Bahrain for providing the financial assistance for presenting this work in Annual Scientific Meeting-2013 of the New Zealand Institute of Medical Laboratory Sciences at Hamilton, New Zealand. Authors are also thankful to the Research and Ethics Committee and the College of Medicine & Medical Sciences, Arabian Gulf University for providing the funds to conduct this research project (Project No. 94). Authors are grateful to Mr. Tomy Kaitharath for his expert technical assistance.

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